DAIDS

VIROLOGY MANUAL

FOR HIV LABORATORIES

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Compiled by

THE DIVISION OF AIDS

NATIONAL INSTITUTE OF ALLERGY & INFECTIOUS DISEASES

NATIONAL INSTITUTES OF HEALTH

and

COLLABORATING INVESTIGATORS

NEUTRALIZING ANTIBODY EVALUATION INFECTIVITY REDUCTION ASSAY

I. PRINCIPLE

The HIV-1 neutralizing antibody assessment by the infectivity reduction assay estimates the reduction of the number of infectious units per milliliter (IUPML) in 1 mL of viral stock dilutions when treated with HIVIG or patient serum. This assay is performed in a 24-well plate and is based on the ACTG consensus plasma quantitative culture assay, using six 5-fold dilutions of viral isolate neutralized by HIVIG, serum or plasma. A 1:125 final concentration of HIVIG is added to each of the dilutions. When autologous serum is used, 1:20 or 1:40 dilution of serum is preferred. Each sample of viral dilution is cocultured with PHA-stimulated normal donor PBMC for 14 days. The supernatant for each individual well is assayed for HIV-1 p24 antigen as a determination of viral growth. The antigen results from each well can be evaluated by a software program to determine the IUPML in the presence of HIVIG, patient serum or normal human serum (control) according to the ACTG Virology Manual. The difference between the IUPML would be used to assess neutralizing capabilities of the test specimen. Based on preliminary studies, assay variation is expected to be 0.7 log.

II. SPECIMEN REQUIREMENTS

Viral stock of autologous or heterologous HIV-1 to be assayed. A pretitered viral stock is not necessary. A virus titration in the presence of normal human serum is included for each virus as part of the assay. (Please see "control set" in Assay Setup below.)

Patient serum/plasma. ACD or CPD-A1 tubes are recommended for plasma collection.

III. REAGENTS

RPMI-1640 with L-glutamine (2 mM) - Store a 4-8^{0}C and note manufacturer's outdate.

Fetal Bovine Serum - Store frozen at -20^{0}C and note manufacturer's outdate. When needed, rapidly that a bottle in a 37^{0}C water bath, then heat-inactivate in a 56^{0}C water bath for 30 minutes with occasional shaking. The level of water in the water bath should be as high as the level of serum in the bottle. Store at 4^{0}C after heat-inactivating for one month.

5% natural, delectinated IL-2 (Boehringer Mannheim, Cellular Products Inc., or Pharmacia). Store at -20^{0}C and note manufacturer's outdate.

0.001% DEAE-Dextran or 2 g/mL polybrene (optional).

Penicillin (100 units/mL)/streptomycin (100 g/mL) or gentamicin (50 g/mL).
Culture Medium - Prepare and store at 4-8^{0}C for up to one month.

RPMI-1640 with glutamine.
20% fetal bovine serum (heat-inactivated).
5% natural, delectinated IL-2 (Boehringer Mannheim, Cellular Products Inc., or Pharmacia).
0.001% DEAE-Dextran or 2 g/mL polybrene (optional).
Penicillin (100 units/mL)/streptomycin (100 g/mL) or gentamicin (50 g/mL).

PHA-stimulated Peripheral Blood Mononuclear Cells (PBMC)--see procedure for Preparation of PHA-stimulated, Uninfected Donor Peripheral Blood Mononuclear Cells (PBMC) in this manual.

NOTE: It is anticipated that the following reagents will be provided centrally by the Virology Quality Assurance (VQA) Laboratory.

Neutralizing Antibody (i.e.,, HIVIG monoclonal antibody).

Pooled Normal Human Sera (NHS).

Primary isolate, MN.

IV. EQUIPMENT AND SUPPLIES

Lab coat.
Gloves.
Laminar flow hood (Class 2 biosafety hood).
Micropipet(s) capable of delivering 20-1000 µL volumes.
Multichannel pipette capable of delivering 20-100 µL volumes.
Disposable sterile pipette tips suitable for the above pipettes (optional).
Disposable reagent reservoirs (optional).
12 x 75 mm sterile, polypropylene tubes.
Sterile 96-well plates (optional).
Sterile 24-well plates (optional).
5 mL polypropylene tubes.
Serological pipettes.
5 x 8 inch low-density polyethylene bags (Nalgene 6255-05088).
Cryovials.
CO_2 incubator (37 ± 1^0C with humidity).
Centrifuge capable of speeds up to 400g and equipped O ring sealed safety cups.
Compound microscope.
Hemacytometer.
Automated cell counter (optional).

V. PROCEDURE

1. Prepare 2 sets of five-fold dilutions of undiluted virus stock (e.g., MN, primary isolate). One set of virus dilutions is the "test set" to which test antibody will be added. The other set of virus dilutions is the "control set" to which control serum will be added.

   Test Set:

   a. Place 1000 µL of undiluted virus into a tube labeled virus stock.

   b. Add 840 µL of culture medium to each of six 12 x 75 mm sterile tubes labeled A to F, or 140 µL into each of six wells if using a 96-well plate for the dilutions.

   c. To tube A, add 210 µL (35 µL for 96-well format) of the viral stock and mix thoroughly. Remove 210 µL (35 µL for 96-well format) from tube A and add to tube B. Repeat this process for a total of six dilutions. The resulting dilution scheme is 1:5 (tube A), 1:25 (tube B), 1:125 (tube C), 1:625 (tube D), 1:3125 (tube E), 1:15625 (tube F). Discard 210 TL (35 µL for 96-well format) from the last dilution (tube F).

   Control Set:

   a. Place 1000 µL of undiluted virus into a tube labeled virus stock.

   b. Add 840 µL of culture medium to each of eight 12 x 75 mm sterile tubes labeled A' to H' (or 35 µL for 96-well format).

   c. To tube A', add 210 µL (or 35 µL for 96-well format) of the viral stock and mix thoroughly. Remove 210 µL from tube A' and add to tube B'. Repeat this process for a total of eight dilutions. The resulting dilution scheme is 1:5 (tube A'), 1:25 (tube B'), 1:125 (tube C'), 1:625 (tube D'), 1:3125 (tube E'), 1:15625 (tube F'), 1:78125 (tube G'), 1:390625 (tube H'). Discard 210 µL (or 35 µL for 96-well format) from the last dilution (tube H').

2. To virus dilution tubes A-F in the "test set" add neutralizing antibody (e.g., HIVIG, monoclonal antibody) at a pre-determined dilution. In the case of HIVIG, add 6.7 µL of undiluted stock HIVIG to the 840 µL already in the tube, for a final dilution of 1:125 (or 1.1 µL to 140 µL if using 96-well format). Add the same concentrations of pooled normal human sera (NHS) to tubes C'-H' in the "control set". Incubate both sets of tubes for 1 hour at 37^0C.

3. Centrifuge 48 hours old PHA stimulated normal donor PBMC at 400 x g for 10 minutes at 20 to 24^0C, remove and discard supernatant. Resuspend cells in culture

medium and enumerate cells. Adjust sample with culture medium to a concentration of 1.25 x 10^6 PBMC/mL.

4. Set up 2 sets of PBMC sterile tubes (5 mL) or wells (24-well or 96-well plate, if you can centrifuge plates), corresponding to the "test set" of virus/HIVIG tubes and the "control test" of virus/NHS tubes.

   a. Add 3.2 mL of above PHA-stimulated PBMC to each tube or 1.6 mL of PBMC to each well (24-well plate) or 160 µL (96-well plate).

   b. Dispense 800 µL of appropriate virus/HIVIG or virus/NHS dilution to corresponding PBMC tubes, or 400 µL to each of two corresponding wells (24-well plate) or 40 µL to each well (96-well plate).

5. Place the tubes or plate into a 5% CO_2, humidified chamber or cover the plate with a 5 x 8 inch low-density polyethylene bag. Incubate overnight the samples at 37^0C. This is day 0.

6. On day 1, after overnight incubation, wash cells 2 times (400 x g for 10 minutes) with culture medium in tubes or wells. If using the well method, after the last wash resuspend in 2 mL (24-wells) or 200 µL (96-wells) of fresh culture medium and return to the corresponding well. If using the tube method, after the last wash resuspend in 4 mL of fresh plasma culture medium and return to the corresponding tube. Transfer cells from corresponding tubes to wells on a 24-well plate (tube A to wells A1, A2; tube B to wells B1, B2, etc.).

7. On day 7, remove one half of the medium (1 mL) or 100 µL if 96-well plates were used and save for future testing (save at -70^0C) and replace with 1 mL of fresh culture medium containing 0.5 x 10^6, 48-hour PHA-stimulated normal donor PBMC or 100 µL if using 96-well plates. Cultures are terminated on day 14, at which time 1.0 mL of medium is withdrawn and stored at -70^0C for subsequent HIV p24 antigen determination.

   For the labs using the 96-well format, the 100 µL harvested either on day 7 or day 14 will be put in 96-well plates and mixed with 100 µL of serum containing media. Cover the plates with sealer and freeze in -70^0C.

   Note: Day 7 culture supernatants should be tested if day 14 shows virus overgrowth (lack of any neutralization).

VI. CALCULATIONS

1. Wells are scored positive if p24 > 30 pg/mL. To calculate infectivity reduction, please enter p24 values (raw data) into virology software to calculate IUPM.

Obtain IUPM for the "control test" (NHS) and for the "test set" (HIVIG). Express infectivity reduction as the *ratio* of IUPM (control) to IUPM (test).

VII. RESULTS

A ten fold reduction equals 90% neutralization.

Note: In preparation for implementing this assay in clinical studies, more discussion will take place regarding neutralization data capture and analysis. Efforts will be made to incorporate the principles of the ID_{50} program developed by John Spouge, M.D. This program calculates virus infectious doses by fitting a model to the data. It obtains log ID50 for the "control set" (NHS) and for the "test set" (HIVIG). Infectivity reduction is expressed as the *difference* between logID_{50} (control) and logID50 (test). One log reduction equals 90% neutralization. For more information about this program, please call Dr. Spouge at 301-496-2475.